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Tunicamycin-Resistant Mutants and Chromosomal Locations of Mutational Sites in *Bacillus subtilis*

SHINTARO NOMURA,[1] * KUNIO YAMANE,[1] TAKASHI SASAKI,† MAKARI YAMASAKI,[2] GAKUZO TAMURA,[2] AND BUNJI MARUO[1]

Institute of Applied Microbiology[1] and Department of Agricultural Chemistry,[2] The University of Tokyo, Bunkyo-ku, Tokyo, 113 Japan

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Two types of tunicamycin-resistant mutants of *Bacillus subtilis* were analyzed, and their mutational sites on the chromosome were mapped. A type 1 mutation that simultaneously expressed hyperproductivity of extracellular α-amylase was located close to *amyE*. Type 2 mutations were near *aroI*.

Tunicamycin (6), an antiviral antibiotic, inhibits the glycosidation of glycoproteins in animal cells (7). In bacteria, the antibiotic inhibits the biosyntheses of peptidoglycan (1, 8, 9) and teichoic acid (2) by preventing the formation of lipid intermediates from uridine diphospho-*N*-acetylmuramylpentapeptide and uridine diphospho-*N*-acetylglucosamine, respectively. Tunicamycin-resistant mutants were isolated from *B. subtilis* NA64 after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. They grew normally in a nutrient broth (3 g of bouillon, 10 g of peptone, and 5 g of sodium chloride per liter at pH 7.0) containing 50 μg of tunicamycin per ml, whereas in the parental strain remarkable morphological changes and lysis were induced by the addition of 0.8 μg of tunicamycin per ml to the medium (4).

The mutants were classified into two types by production of extracellular α-amylase. Type 1, B7, produced five times as much extracellular α-amylase as NA64 did, and type 2 mutants, B4, B8, B9,and B10, showed production of extracellular α-amylase almost equal to that of NA64 (Table 1). All of the mutants had the same production of extracellular protease and ribonuclease and the same sensitivity to other antibiotics as their parental strain. The chromosomal locations of the mutational sites for the two types of tunicamycin-resistant characters are described in this paper.

Strains used are listed in Table 1. PBS1-mediated transduction was performed by a slightly modified method of Young et al. (14). All of the lysates used showed 20 to 30% cotransduction of *lys-21* with *metB5*. Transforming DNA was prepared by the method of Saito and Miura (3), and transformation was performed by the method of Yoshikawa (13). In each transformation experiment, less than 0.05 μg of transforming DNA per ml was used. The frequencies of double transformation of two independent markers were less than 0.8%. The tunicamycin-resistant character (Tm^r) in transductants and transformants was determined on nutrient agar plates containing 5 μg of tunicamycin per ml. Mapping results in transduction and transformation experiments in the figures were expressed as percentages of recombination by the following conversion: recombination = 1 − cotransfer.

The α-amylase structural gene (*amyE*) was mapped near the *aroI* locus by Yuki (16) and Steinmetz et al. (5). A regulator gene (*amyR*) is closely linked with *amyE* (10, 15). To characterize the genetic relation of the hyperproductivity of α-amylase to Tm^r in B7, DNA from B7 was transferred into M07-2-31 by DNA-mediated transformation. Tm^r and Aro^+ transformants were separately isolated (Table 2). Tm^r was cotransformable with $amyE^+$ at an average frequency of 90% and with $aroI^+$ at 8%. $aroI^+$ was cotransformable with $amyE^+$ and Tm^r at frequencies of about 35 and 8%, respectively. These results implied an order of *tmr-7-amyE07-aroI906* (Fig. 1). All of the transformants that acquired *tmr-7* and $amyE^+$ produced about 200 U of extracellular α-amylase per ml, as B7 did. Thus, the tunicamycin resistance and hyperproduction of the enzyme found in B7 were due to a single mutation, as suggested previously (4). Yamaguchi et al. (11) reported that the cotransformation frequency of *amyR* with *amyE07* was at least 96.1%. *tmr-7* seemed to be not included in *amyR*, because the cotransformation frequency of *tmr-7* with *amyE07* was 90% and because *tmr-7 amyR1* transformants were isolated at a frequency of about 4% when B7 DNA was transferred into NA20-22 and Tm^r transformants were selected.

† Present address: Research Laboratories of Sapporo Breweries Ltd., Mita 1-4-1, Meguro-ku, Tokyo, 153 Japan.

TABLE 1. *B. subtilis strains used and their production of extracellular α-amylase*

Strain	Genotype	α-Amylase production (U/ml)
M07-2-31	*aroI906 metB5 amyR2 amyE07*[a]	0
NA20-22	*metB5 trpB3 amyR1 amyE*$^+$	10
NA64	*purB6 metB5 amyR2 amyE*$^+$	40
B4	*purB6 metB5 amyR2 amyE*$^+$ *tmr-4*	50
B7	*purB6 metB5 amyR2 amyE*$^+$ *tmr-7*	210
B8	*purB6 metB5 amyR2 amyE*$^+$ *tmr-8*	45
B9	*purB6 metB5 amyR2 amyE*$^+$ *tmr-9*	40
B10	*purB6 metB5 amyR2 amyE*$^+$ *tmr-10*	40
LTR-7	*lin-2 amyR2 amyE*$^+$ *tmr-7*	250

[a] The strain carrying *amyE07* produces cross-reacting material with a rabbit antiserum against *B. subtilis* α-amylase (11, 12).

TABLE 2. *Three-point transformation crosses involving aroI906, amyE07, and tmr-7 or tmr-8 markers*

Recipient genotype	Donor genotype	Recombinant		
		Selection	Class	No.
aroI906 metB5 amyE07 amyR2 (M07-2-31)	*purB6 metB5 amyE*$^+$ *amyR2 tmr-7* (B7)	Tm^r	Tm^r Amy$^+$ Aro$^+$	30
			Tm^r Amy$^+$ Aro$^-$	319
			Tm^r Amy$^-$ Aro$^+$	0
			Tm^r Amy$^-$ Aro$^-$	41
		Aro$^+$	Aro$^+$ Amy$^+$ Tm^r	34
			Aro$^+$ Amy$^+$ Tm^s	160
			Aro$^+$ Amy$^-$ Tm^r	2
			Aro$^+$ Amy$^-$ Tm^s	403
	purB6 metB5 amyE$^+$ *amyR2 tmr-8* (B8)	Tm^r	Tm^r Amy$^+$ Aro$^+$	131
			Tm^r Amy$^+$ Aro$^-$	15
			Tm^r Amy$^-$ Aro$^+$	198
			Tm^r Amy$^-$ Aro$^-$	10
		Aro$^+$	Aro$^+$ Amy$^+$ Tm^r	88
			Aro$^+$ Amy$^+$ Tm^s	8
			Aro$^+$ Amy$^-$ Tm^r	139
			Aro$^+$ Amy$^-$ Tm^s	11

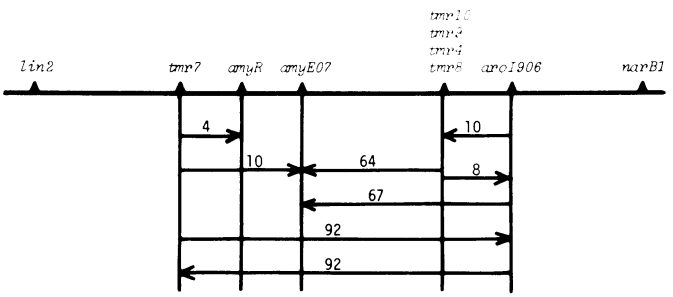

FIG. 1. *Genetic map of the tmr-7-to-aroI906 region, determined by DNA-mediated transformation. Distances are expressed as percentages of recombination. Arrows point from selected to unselected markers.*

tmr-8 showed an extremely high linkage with *aroI* in DNA-mediated transformation (Table 2). The order of genetic markers around *aroI* was *amyE07-tmr-8-aroI906. tmr-4, tmr-9,* and *tmr-10* were also mapped at about the same position as that of *tmr-8.* These mutations seemed to have occurred at different sites in the same gene. Three percent of the tunicamycin-sensitive recombinants in Aro$^+$ transformants were obtained from the transformation of a type

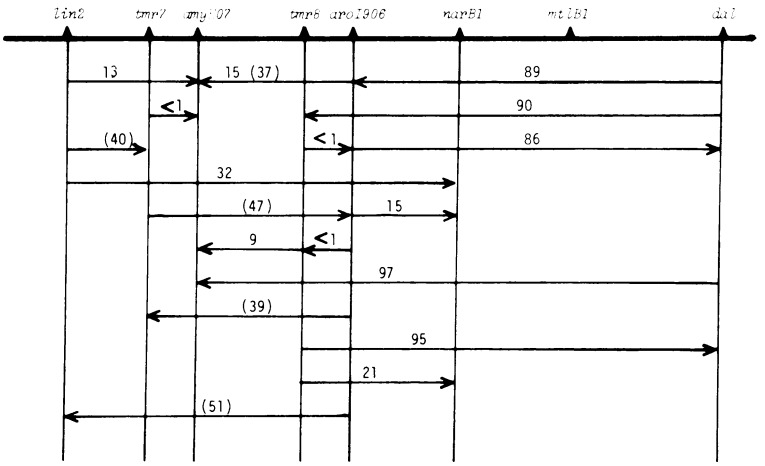

FIG. 2. *Genetic map of the lin-2-to-dal region with PBS1-mediated transduction. Distances are expressed as percentages of recombination. Arrows point from selected to unselected markers. Values in parentheses were obtained by PBS1 lysates made on B7 and LTR-7.*

1 strain, *tmr-7 aroI906,* by the DNA from a type 2 strain, *tmr-8 aroI*$^+$.

The loci for *tmr-7* and *tmr-8* were further confirmed by transduction with PBS1 (Fig. 2). *tmr-7* and *tmr-8* did not segregate from *amyE*$^+$ and *aroI*$^+$, respectively.

Steinmetz et al. (5) reported that the cotransduction frequency of *aroI* with *amy* $^+$ was 92% . A comparable frequency, 85%, between these markers was observed by lysates of PBS1 on NA64 and B8. Higher values, however, were obtained in the same region and in the region including the *aroI* to *tmr-7* markers when PBS1 lysates on LTR-7 and B7 were used (shown in parentheses in Fig. 2). The phage did not seem to contain host DNA of abnormal length, because the cotransduction frequencies of *lys-21* with *metB5* were about 25%. The localization of other genetic markers in the *lin-2*-to-*dal* region determined by transduction are also included in Fig. 2 (strains used are not shown).

Another tunicamycin-resistant mutant, which grew in nutrient broth containing 25 μg of tunicamycin per ml, was isolated. Genetic characterization of this mutant was not successful, since the resistance to tunicamycin was not due to a single mutation.

These results indicate the presence of at least two different chromosomal loci for the phenotypic tunicamycin resistance character. It seems noteworthy that hyperproduction of extracellular α-amylase occurs only in the type 1 tunicamycin resistance mutation. To understand the function of this antibiotic in *B. subtilis,* whether the resistant character is directly related to the prevention of inhibition of lipid intermediate synthesis is now under investigation.

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LITERATURE CITED

1. **Bettinger, G. E., and F. E. Young.** 1975. Tunicamycin, an inhibitor of *Bacillus* peptidoglycan synthesis. A new site of inhibition. Biochem. Biophys. Res. Commun. **67**:16-21.
2. **McArthur, H. A. I., F. M. Roberts, I. C. Honcock, and J. Baddiley.** 1978. Lipid intermediate in the biosynthesis of the linkage unit between teichoic acid and peptidoglycan. FEBS Lett. **86**:193-200.
3. **Saito, H., and K. Miura.** 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys Acta **72**:619-629.
4. **Sasaki, T., M. Yamasaki, B. Maruo, Y. Yoneda, K. Yamane, A. Takatsuki, and G. Tamura.** 1976. Hyperproduction of extracellular α-amylase by a tunicamycin resistant mutant of *Bacillus subtilis.* Biochem. Biophys. Res. Commun. **70**:125-130.
5. **Steinmetz, M., F. Kunst, and R. Dedonder.** 1976. Mapping of mutations affecting synthesis of exocellular enzymes in *Bacillus subtilis.* Mol. Gen. Genet. **148**:281-285.
6. **Takatsuki, A., K. Arima, and G. Tamura.** 1971. Tunicamycin, a new antibiotic. I. Isolation and characterization of tunicamycin. J. Antibiot. **24**:215-223.
7. **Takatsuki, A., K. Kohno, and G. Tamura.** 1975. Inhibition of biosynthesis of polyisoprenol sugars in chick embryo microsomes by tunicamycin. Agric. Biol. Chem. **39**:2089-2091.
8. **Tamura, G., T. Sasaki, M. Matsuhashi, A. Takatsuki, and M. Yamasaki.** 1976. Tunicamycin inhibits the formation of lipid intermediate in cell free peptidoglycan synthesis of bacteria. Agric. Biol. Chem. **40**:447-449.
9. **Ward, J. B.** 1977. Tunicamycin, inhibition of bacterial wall polymer synthesis. FEBS Lett. **78**:151-154.
10. **Yamaguchi, K., H. Matsuzaki, and B. Maruo.** 1969. Participation of a regulator gene in the α-amylase production of *Bacillus subtilis.* J. Gen. Appl. Microbiol. **15**:97-107.
11. **Yamaguchi, K., Y. Nagata, and B. Maruo.** 1974. Iso-

lation of mutants defective in α-amylase from *Bacillus subtilis*. J. Bacteriol. **119**:416–424.

12. **Yamane, K., K. Yamaguchi, and B. Maruo.** 1973. Purification and properties of a cross-reacting material related to α-amylase and biochemical comparison with the parent α-amylase. Biochim. Biophys. Acta **259**:323–340.
13. **Yoshikawa, H.** 1970. Temperature sensitive mutant of *Bacillus subtilis*. I. Multiforked replication and sequential transfer of deoxyribonucleic acid by a temperature sensitive mutant. Proc. Natl. Acad. Sci. U.S.A. **65**:206–213.
14. **Young, F. E., C. Smith, and B. E. Reilly.** 1969. Chromosomal location of genes regulating resistance to bacteriophage in *Bacillus subtilis*. J. Bacteriol. **98**:1087–1097.
15. **Yuki, S.** 1968. On the gene controlling the rate of α-amylase production in *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **31**:182–187.
16. **Yuki, S.** 1975. Chromosomal location of the structural gene for α-amylase in *Bacillus subtilis*. Jpn. J. Genet. **50**:155–157.